

# Footprinting protein–DNA complexes using the hydroxyl radical

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**Hydroxyl radical footprinting has been widely used for studying the structure of DNA and DNA–protein complexes. The high reactivity and lack of base specificity of the hydroxyl radical makes it an excellent probe for high-resolution footprinting of DNA–protein complexes; this technique can provide structural detail that is not achievable using DNase I footprinting. Hydroxyl radical footprinting experiments can be carried out using readily available and inexpensive reagents and lab equipment. This method involves using the hydroxyl radical to cleave a nucleic acid molecule that is bound to a protein, followed by separating the cleavage products on a denaturing electrophoresis gel to identify the protein-binding sites on the nucleic acid molecule. We describe a protocol for hydroxyl radical footprinting of DNA–protein complexes, along with a troubleshooting guide, that allows researchers to obtain efficient cleavage of DNA in the presence and absence of proteins. This protocol can be completed in 2 d.**

## INTRODUCTION

Footprinting is a widely used method for delineating the binding site of a protein or small molecule on DNA or RNA<sup>1–4</sup>. A protein that is bound to a specific DNA sequence shields the DNA duplex from attack through an enzyme or chemical reagent, which is otherwise free to react with the unbound portions of the DNA molecule. The binding site of the protein is revealed as the DNA nucleotides that are not cleaved by the chemical or enzyme. In practice, the cleavage pattern of a protein–DNA complex is compared to the cleavage pattern of the same DNA molecule not bound by protein. The cleavage pattern is visualized as a ladder of bands after denaturing gel electrophoresis of singly end-labeled DNA. The missing (or diminished) bands in the lane that is derived from the cleavage of the nucleic acid–protein complex constitute the footprint. This is shown schematically in **Figure 1**, and **Figure 2** shows the results of an actual footprinting experiment.

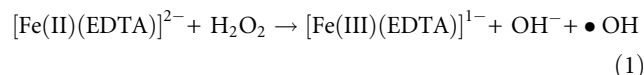
## DNase I footprinting

Among the earliest footprints were those produced through digestion of a DNA–protein complex with the nonspecific endonuclease deoxyribonuclease I (DNase I)<sup>5</sup>. DNase I footprints are easy to produce and so provide a convenient way to locate the binding site of a protein on DNA<sup>6</sup>. As a footprinting reagent, DNase I does suffer from a few limitations. The enzyme, while not sequence-specific, exhibits preferences (likely based on local DNA structural heterogeneity) as to where it cuts duplex DNA<sup>7</sup>. The result is that DNase I does not cleave all nucleotides in a particular DNA molecule, and so only a subset of the potential protein-binding sites can be interrogated in a footprinting experiment. DNase I is relatively large, comparable in size to the DNA-binding proteins that are studied in a footprinting experiment. DNase I footprints can therefore overestimate the size of the binding site of a protein<sup>8</sup>. Because DNase I must itself bind to the DNA molecule before cleaving it, a DNA-binding protein can interfere with enzyme binding and so inhibit cleavage of nucleotides that are not actually covered by the DNA-binding protein.

## Hydroxyl radical footprinting

Structural interactions in a DNA–protein complex can be better understood by carrying out footprinting using the small but extremely reactive hydroxyl radical<sup>9–11</sup>. The hydroxyl radical cleaves the DNA strand by abstracting a hydrogen atom from a deoxyribose sugar in the DNA backbone<sup>12</sup> (**Fig. 3**). Hydroxyl radical footprinting has proven to be of great use in the study of DNA<sup>13–16</sup> and RNA<sup>17</sup> structures, and in understanding the interaction of DNA with various ligands, including proteins<sup>10</sup> and small molecules<sup>2,18,19</sup>. A major advantage of hydroxyl radical footprinting is that it yields base- and sequence-independent DNA cleavage at single-nucleotide resolution<sup>10,20</sup>.

The hydroxyl radical footprinting technique uses commonly available lab equipment and inexpensive reagents. Hydroxyl radicals are generated by the Fenton reaction<sup>21,22</sup> in which [Fe(II)(EDTA)]<sup>2–</sup> reacts with hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), thereby producing the hydroxyl radical (•OH) and [Fe(III)(EDTA)]<sup>1–</sup> (Eq. 1):



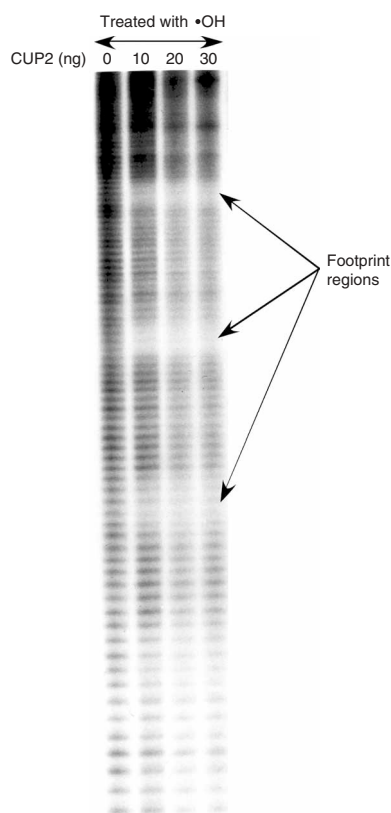
Sodium ascorbate is added to the reaction mixture to reduce the [Fe(III)(EDTA)]<sup>1–</sup> product back to [Fe(II)(EDTA)]<sup>2–</sup>, thereby making the system catalytic in iron and reducing the amount of starting Fe(II) that is required. In a standard hydroxyl radical footprinting experiment, a DNA molecule radiolabeled with <sup>32</sup>P at one end of one strand is treated with the hydroxyl radical in the presence or absence of a DNA-binding protein. Cleavage products are separated by denaturing PAGE and visualized using a phosphorimager screen. Generation of the hydroxyl radical by the Fenton reaction is fast, so the cleavage reaction can be carried out in <5 min. In fact, the Fenton reaction is so rapid that recent reports demonstrate that millisecond time resolution can be achieved for hydroxyl radical footprinting using a standard fast-kinetics device, opening the way for time-resolved hydroxyl radical footprinting experiments in the laboratory<sup>23,24</sup>.

## PROTOCOL

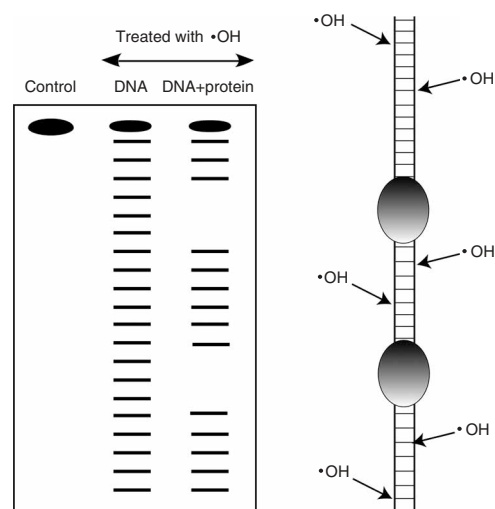
**Figure 1** | Correspondence of the structure of a DNA–protein complex (right) and the hydroxyl radical footprint as imaged by an electrophoresis gel (left). The protein (shaded ovals) binds to two separate sites in the DNA molecule. The hydroxyl radical ( $\bullet\text{OH}$ ) is unable to cleave the DNA molecule at sites where the protein is covering the DNA; the remainder of the DNA is accessible to  $\bullet\text{OH}$  and is cleaved. Cleavage results in bands on the gel. The control lane of the gel (left) contains untreated DNA. The other two lanes show DNA that was treated with the hydroxyl radical in the absence (center) and presence (right) of protein. Cleavage bands are absent at nucleotides that are covered by the protein, showing clearly where the protein binds to DNA. The single-nucleotide resolution of the hydroxyl radical cleavage pattern provides a high-resolution footprint.

Hydroxyl radical footprinting has been used to study protein–DNA<sup>25,26</sup> and protein–RNA<sup>3</sup> complexes *in vivo*, using  $\gamma$  radiation or synchrotron radiation as the source of hydroxyl radicals.

Hydroxyl radical footprinting also can be performed using fluorescently labeled nucleic acids<sup>23,27,28</sup>, using the PCR in conjunction with a fluorophore-tagged primer to generate an end-labeled DNA molecule. Using fluorophore-tagged DNA for footprinting offers several advantages, including eliminating the use of radioactivity and compatibility with rapid electrophoretic separation using a capillary electrophoresis device. Drawbacks are the expense of the necessary instrumentation and the fact that the end of the DNA is chemically modified by the attached fluorophore. Radiolabeling of nucleic acids remains the method of choice for footprinting studies due to its high sensitivity and economical use of existing laboratory equipment and methods. The main



**Figure 2** | Hydroxyl radical footprint of the CUP2 protein bound to a 154-bp restriction fragment. Lane designations (from left): DNA in the absence of CUP2 protein (0), and in the presence of 10, 20 and 30 ng of CUP2, respectively. Strong protection is observed at TC(T)<sub>4-6</sub> GCT sequences, which are sites where CUP2 binds to DNA. Reproduced with permission from ref. 59.



advantages and limitations of hydroxyl radical footprinting are summarized in **Box 1**.

### Alternative methods

Structural characterization of protein–DNA complexes can be carried out using techniques other than hydroxyl radical footprinting.

**X-ray crystallography.** The major advantage of X-ray crystallography is that it provides a view at atomic resolution of the interactions within protein–DNA complexes<sup>29,30</sup>. Drawbacks of this technique are the necessity for growth of diffraction-quality crystals and the requirement of a dedicated lab with expensive instrumentation.

**Electrophoretic mobility shift assay.** This method has been widely used for qualitative and quantitative detection of protein–nucleic acid complexes<sup>31–36</sup>. Electrophoretic mobility shift assay (EMSA) is based on the principle that the electrophoretic mobility of a protein–DNA complex is lower than that of free DNA. One limitation of EMSA is that the protein–DNA complex is not necessarily at chemical equilibrium at the time of electrophoresis. A further limitation is that the precise nucleotide sequence of the protein-binding site is not easy to determine using EMSA.

**Nitrocellulose filter-binding assay.** Although no longer widely used, this is a simple and rapid method for detecting protein binding to DNA or RNA<sup>37–40</sup>. Filter binding is based on the observation that protein binds to nitrocellulose whereas nucleic acids do not. When a sample containing DNA and protein is filtered through nitrocellulose, any DNA molecules that are bound to protein will be retained on the filter and can subsequently be analyzed. Nitrocellulose filter binding, such as EMSA, is a non-equilibrium technique. Like EMSA, nitrocellulose filter binding does not permit easy identification of the precise nucleotide sequence of the protein-binding site.

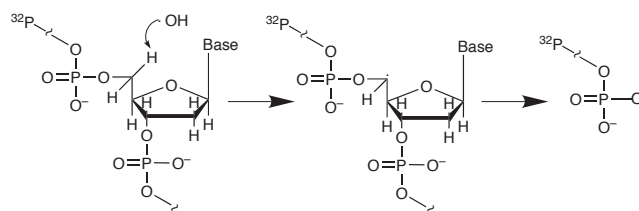
### Experimental design

Here, we describe the protocol for hydroxyl radical footprinting of a protein–DNA complex, based on methods routinely used in

our lab<sup>9,10,41–53</sup>. This protocol begins with hydroxyl radical cleavage of an equilibrated mixture of the protein and DNA. After cleavage, protein is removed by phenol:chloroform extraction. The DNA cleavage products are ethanol precipitated and then subjected to electrophoresis on a denaturing polyacrylamide gel. After electrophoresis, the gel is dried and imaged by exposure to a phosphor screen (see Fig. 2).

**Preparation of DNA and protein.** The hydroxyl radical footprinting experiment requires a linear DNA molecule that contains the binding site for the protein. A DNA molecule 50–100 bp in length, with the protein-binding site near the center, gives optimal footprinting patterns on a typical denaturing gel, but other DNA lengths may be used if the gel percentage is changed (see Table 1). The DNA molecule must be radiolabeled at the 5' end of one strand with <sup>32</sup>P. The KinaseMax kit (Ambion) is used to radiolabel the DNA. There are several possible sources of the DNA molecule. The classic approach is to prepare plasmid DNA containing the protein-binding site. The plasmid is treated using a single-cutting restriction endonuclease, and the 5' ends of the DNA are then labeled with <sup>32</sup>P. Treatment with a second restriction endonuclease releases the DNA fragment of interest, now containing only one radiolabel. The desired radiolabeled DNA molecule is purified using native PAGE. A more convenient method of preparation is to use the PCR to amplify the desired DNA sequence, using one primer with a 5'-<sup>32</sup>P end label. Radiolabeled DNA should not be stored for long periods of time (> 1 month) due to loss of activity of <sup>32</sup>P and potential degradation of the DNA. The protein of interest is purified in advance of the footprinting experiment, using protocols appropriate for that protein.

**Optimization of the protein–DNA binding reaction.** The conditions for binding the protein to DNA will be specific to the system under investigation. The starting point should be the conditions (buffer, temperature, protein concentration) that have been found previously under which the protein binds avidly to DNA (as assayed, e.g., by EMSA<sup>36</sup>). The binding buffer must then be optimized for footprinting. One commonly needed modification is the removal (or minimization) of glycerol in the binding buffer, because glycerol inhibits the hydroxyl radical cleavage reaction<sup>9</sup>.



**Figure 3** | Details of the chemistry of the hydroxyl radical with the deoxyribose backbone of DNA. The major reaction of the hydroxyl radical with the DNA backbone is to abstract a deoxyribose 5'-hydrogen atom<sup>60</sup>, leading to the formation of a free radical intermediate, which undergoes backbone cleavage to form products. Only the radiolabelled product is shown.

Glycerol is commonly added to buffers to eliminate the formation of ice crystals upon storage of a protein solution at –20 °C, and so is not necessary for footprinting experiments, which are conducted at temperatures > 0 °C.

**Optimization of the hydroxyl radical cleavage reaction.** Conditions must be found for the hydroxyl radical reaction (by varying the duration of the reaction or the concentrations of the cleavage reagents) such that each molecule of DNA is cleaved at most one time (single-hit kinetics). This condition is satisfied when ~ 70% of the DNA in the sample remains uncut<sup>6</sup>. If this condition is not satisfied, quantitation of the electrophoretic pattern will be difficult or impossible because the occurrence of multiple cleavage events within a single DNA molecule artificially biases the product distribution toward smaller DNA fragments<sup>54</sup>. The extent of cleavage is judged by the integrated intensity of the full-length DNA band at the top of the gel lane containing a footprinting sample, compared to the full-length DNA band in a control sample containing the same amount of untreated DNA. The integrated intensity of the full-length band for the footprinted sample should be 60–70% of the intensity of the full-length band in the control sample.

**Controls.** Important control samples that must be electrophoresed alongside a footprinting sample are

- (1) Intact DNA that has been mock-treated in the presence as well as in the absence of protein. This control is important to ensure

## BOX 1 | ADVANTAGES AND LIMITATIONS OF HYDROXYL RADICAL FOOTPRINTING

### Advantages

- Hydroxyl radical footprinting can be used to study the structure of a protein–DNA complex at single-nucleotide resolution, in solution, under equilibrium conditions.
- The hydroxyl radical is a very reactive and nonselective free radical, so DNA cleavage is nonspecific with respect to nucleotide sequence.
- Experiments can be carried out using inexpensive reagents and commonly available lab equipment.
- Footprinting experiments can be carried out and results can be obtained within a span of 2 d.

### Limitations

- Optimization of the cleavage reaction for a particular protein–DNA complex can be time-consuming.
- More than one of the deoxyribose hydrogen atoms of DNA is subject to abstraction by the hydroxyl radical<sup>12,60</sup>. This leads to the formation of multiple cleavage products at a particular nucleotide (Fig. 4), which can pose a challenge to quantitation, but only for very high-resolution electrophoresis patterns (see, ANTICIPATED RESULTS).
- Although fine structural details of a protein–DNA complex can be derived from a hydroxyl radical footprint<sup>41,42</sup>, a high-resolution 3D structure, such as is obtained from X-ray crystallography and NMR spectroscopy, cannot be achieved.

## PROTOCOL

that the input DNA is not degraded before the footprinting experiment. The same amount of a control DNA sample (as judged by radioactivity) as the footprinted sample must be loaded on the gel. The control DNA sample must exhibit a single, intense band that runs with a mobility characteristic of the full-length DNA strand (i.e., near the top of the gel). No evidence of shorter DNA fragments must be present in the gel lane. If shorter DNA fragments are seen, this indicates that the DNA is degraded, and it will be difficult or impossible to discern a footprint in the experimental sample. If degraded DNA is found, a new DNA sample must be prepared.

- (2) DNA treated with the hydroxyl radical in the absence of protein. This control sample provides the baseline pattern of hydroxyl radical cleavage for that DNA sequence. The footprint of the protein is found where the cleavage pattern of the experimental footprinting sample differs from that of this control sample (see **Figs. 1** and **2**).

## MATERIALS

### REAGENTS

- Plasmid DNA, which contains the binding site for the protein of interest inserted into a cloning site, so that a restriction fragment containing the binding site can readily be prepared and radiolabeled at the 5' end of one strand by standard methods<sup>55</sup> (see EXPERIMENTAL DESIGN)  
*Note:* Radiolabeled DNA should be prepared in advance and frozen at  $-20^{\circ}\text{C}$ , but should not be stored for longer than 1 month.
- Solution containing the DNA-binding protein
- Binding buffer (specific for each protein)
- KinaseMax kit (Ambion, cat. no. 1520)
- Adenosine-5'-triphosphate,  $\gamma$ - $^{32}\text{P}$  (Perkin-Elmer, cat. no. BLU 002A)  
**! CAUTION** Use safety goggles, lab coat and a Plexiglas shield when working with  $^{32}\text{P}$ . Institutional guidelines for working with radioactive materials must be followed, including laboratory certification.
- Ammonium iron(II) sulfate hexahydrate  $[(\text{NH}_4)_2\text{Fe}(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}]$  (Sigma-Aldrich, cat. no. 203505)
- EDTA disodium salt dihydrate ( $\text{Na}_2\text{EDTA} \cdot 2\text{H}_2\text{O}$ ) (Fisher, cat. no. BP120-500)
- $\text{H}_2\text{O}_2$  30% wt/vol (Fisher, cat. no. MHX06351) (see REAGENT SETUP)
- L(+)-ascorbic acid sodium salt (Sigma, cat. no. A7631)
- Milli-Q purified water or equivalent, autoclaved. Purified water is indicated in this protocol by  $\text{dH}_2\text{O}$  **! CRITICAL** The quality of water used in the preparation of reagents and buffers is very important. Organic contaminants in the water can interfere with the hydroxyl radical cleavage reaction, as can contaminating metal ions (such as iron or copper).
- Tris base
- Hydrochloric acid (HCl, concentrated) **! CAUTION** Corrosive and can be fatal if swallowed. Proper ventilation, a fume hood and personal protection (gloves, lab coat and safety goggles) must be used when handling concentrated HCl solutions.
- Sodium hydroxide (NaOH) **! CAUTION** Highly corrosive. Gloves, lab coat and safety goggles must be used when handling NaOH solutions.
- Sodium acetate
- Ethanol (100 and 70% vol/vol), chilled to  $-20^{\circ}\text{C}$
- Thiourea **! CAUTION** Potential carcinogen and may cause irritation to the eyes, skin and respiratory tract. Wear gloves, lab coat and safety goggles when handling thiourea solution.
- TEMED (*N,N'*-tetramethylethylenediamine) (Sigma, cat. no. T9281).  
**! CAUTION** Wear gloves, lab coat and safety goggles when handling TEMED solution.
- Ammonium persulfate (APS; Fisher, cat. no. BP 179) (see REAGENT SETUP)
- Urea
- Acrylamide monomer (Sigma, cat. no. A3553) **! CRITICAL** Store at  $4^{\circ}\text{C}$  and protect from light. **! CAUTION** Acrylamide is a neurotoxin. Wear gloves, lab coat and safety goggles when handling acrylamide. Preparation of acrylamide solutions must be carried out in the hood.

**TABLE 1** | Concentration of acrylamide giving optimum resolution for footprinting.

Acrylamide (%)	DNA fragment sizes (bp) that are well resolved
30	2–8
20	8–25
10	25–35
8	35–45
6	45–70
5	70–300

- (3) The products of a Maxam–Gilbert guanine-specific sequencing reaction<sup>55</sup> that was performed on the DNA molecule of interest, to serve as size markers to aid in identifying the nucleotides that make up the protein-binding site.

- *N,N'*-bisacrylamide (Sigma, cat. no. M2022) **! CRITICAL** Store at  $4^{\circ}\text{C}$  and protect from light. **! CAUTION** Bisacrylamide is a neurotoxin. Wear gloves, lab coat and safety goggles when handling bisacrylamide solutions. Preparation of bisacrylamide solutions must be carried out in the hood.
- Sigmacote (Sigma, cat. no. SL-2)
- Iron(II)EDTA (see REAGENT SETUP)
- Sodium ascorbate (see REAGENT SETUP)
- Stop buffer (see REAGENT SETUP)
- Tris–EDTA (TE) buffer (see REAGENT SETUP)
- Tris–borate–EDTA (TBE) buffer (10 $\times$ ) (see REAGENT SETUP)
- 40% Gel solution (38% acrylamide, 2% bisacrylamide) (see REAGENT SETUP)
- Polyacrylamide gel mixture (see REAGENT SETUP)

### EQUIPMENT

- SpeedVac concentrator (model no. DNA 120; Savant)
- Pipettors, 1–10, 2–20, 20–200 and 200–1,000  $\mu\text{l}$  (models P10, P20, P200 and P1000, respectively; Gilson), and corresponding tips
- Gel-loading tips, 0.4 mm (Rainin, cat. no. GT-250-4)
- Vertical gel electrophoresis apparatus for running thin sequencing (denaturing) gels (e.g., model no. S3S T-Rex Aluminum Backed Sequencer; Owl Separation Systems)  
*Note:* The larger of the two glass gel plates must be siliconized by applying 1 ml of Sigmacote to the plate using a Kimwipe. Siliconization prevents the gel from sticking to the plate during transfer to chromatography paper following electrophoresis.
- Side-arm flask and stopper, and access to laboratory vacuum or a water aspirator
- Eppendorf microcentrifuge (model no. 5415 C)
- Spacer set and 30-well comb (0.33 mm thick)
- Power supply ( $>2,500$  V) (PowerPac HV; Bio-Rad, cat. no. 164-5056)
- Saran wrap
- Chromatography paper (Whatman, cat. no. 3030917)
- Storm 860 Phosphorimager, including storage phosphor screens, exposure cassettes, and ImageQuant software (GE Healthcare, cat. no. 63-0035-62)
- SAFA gel analysis software<sup>56</sup>. The software and documentation are freely available for download at <http://safa.stanford.edu>

### REAGENT SETUP

**Iron(II)EDTA** Prepare a 20 mM iron(II) stock solution by dissolving 40 mg of  $(\text{NH}_4)_2\text{Fe}(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$  in 5 ml of  $\text{dH}_2\text{O}$ . Mix equal volumes of the 20 mM iron(II) stock solution and 40 mM EDTA. (A twofold excess of EDTA is used to ensure that all iron(II) is bound to EDTA.) Dispense 1 ml aliquots of this solution into 1.5-ml Eppendorf tubes covered in aluminum foil. Store tubes in a  $-20^{\circ}\text{C}$  freezer. Before use in the cleavage reaction, bring a tube of iron(II)(EDTA) stock solution to room temperature ( $21$ – $25^{\circ}\text{C}$ ). Dilute this stock solution with the appropriate amount of  $\text{dH}_2\text{O}$  to prepare a solution of iron(II)EDTA of the appropriate concentration for the footprinting reaction

(e.g., dilute the stock solution 10:1 to prepare the 1 mM Fe(II)/2 mM EDTA solution used in the standard protocol). **▲ CRITICAL** Avoid exposing the iron(II)EDTA solution to light. This solution is colorless; orange-colored solutions and/or solutions with a precipitate should be discarded.

**Sodium ascorbate** Prepare a 10 mM solution by dissolving 20 mg of sodium ascorbate in 10 ml of dH<sub>2</sub>O. Dispense 1 ml aliquots of this solution into 1.5-ml Eppendorf microcentrifuge tubes wrapped in aluminum foil. Store at -20 °C. Thaw a tube of ascorbate solution before using. **▲ CRITICAL** Mix thoroughly and take care to protect the solution from light. Use a fresh tube of ascorbate solution each day. **H<sub>2</sub>O<sub>2</sub>** Prepare a 0.6% wt/vol H<sub>2</sub>O<sub>2</sub> solution by adding 98 μl of dH<sub>2</sub>O to 2 μl of 30% wt/vol H<sub>2</sub>O<sub>2</sub>. **▲ CRITICAL** Protect the solution from light by covering with aluminum foil. Discard the hydroxyl radical reagents [iron(II)EDTA, H<sub>2</sub>O<sub>2</sub>, sodium ascorbate] after use. New reagent solutions should be used each day, by thawing frozen stock solutions.

**Stop buffer** Prepare a 100 mM thiourea solution by dissolving 7.6 mg of solid thiourea into 1.0 ml of dH<sub>2</sub>O. Store at 4 °C.

**TE buffer** Prepare 10 mM Tris-HCl, 0.1 mM EDTA (pH 8.0)

**TBE buffer (10×)** Mix 109 g Tris base, 55 g boric acid and 40 ml 0.5 M EDTA (pH 8.0). Bring the volume to 1 l by adding dH<sub>2</sub>O.

**APS (10% wt/vol)** Dissolve 100 mg of APS in 1 ml dH<sub>2</sub>O.

**Gel-loading dye (2×)** Dissolve 5 mg of bromphenol blue, 5 mg of xylene cyanol, 4.8 g urea and 186 mg EDTA in 10 ml dH<sub>2</sub>O.

**40% Gel solution (38% acrylamide, 2% bisacrylamide)** Mix 38 g of acrylamide and 2 g of bisacrylamide in a 500-ml beaker. Add dH<sub>2</sub>O to bring the volume of the slurry to 90 ml. Stir until the solution becomes clear and then bring the final volume to 100 ml. Protect the solution from light by covering the beaker with aluminum foil. **! CAUTION** Weighing of acrylamide and

**TABLE 2** | Components of denaturing polyacrylamide gel mixtures.

	5% Gel	10% Gel	20% Gel
40% Gel solution (ml)	12.5	25	50
10× TBE buffer (ml)	10	10	10
Urea (g)	42	42	42
dH <sub>2</sub> O (ml)	42.5	30	5
Polymerize by adding			
APS (10%) (ml)	1.0	0.75	0.5
TEMED (μl)	100	75	50

APS, ammonium persulfate; TBE, Tris-borate-EDTA; TEMED, *N,N'*-tetramethylethylenediamine.

bisacrylamide, as well as solution preparation, must be carried out in the hood while wearing goggles, gloves, lab coat and a mask.

**Polyacrylamide gel mixture** Prepare the mixture for a denaturing polyacrylamide gel containing 7 M urea. **Table 2** shows the amount of each component that is needed to prepare gels of various percentages, based upon a final volume of 100 ml of gel solution. The acrylamide gel solution must be vacuum filtered and degassed before initiating polymerization. The percentage of acrylamide used in the gel solution depends upon the distance of the protein-binding site from the labeled end of the DNA molecule (**Table 1**). For example, if the protein-binding site is located 50 bp from the 5' end of the radiolabeled DNA strand, a 6% gel will provide good resolution of cleavage products in the region of the binding site.

## PROCEDURE

### Hydroxyl radical footprinting reaction ● TIMING day 1, 1–2 h

**1** | To prepare an experimental footprinting sample, place ~10 kc.p.m. of singly end-labeled DNA containing the protein-binding site, the required amount of purified protein, and 0.5 μg of calf thymus DNA, in a total volume of 10 μl of binding buffer, in a 1.5-ml Eppendorf microcentrifuge tube. The molar amount of purified protein should be equal to or greater than the molar amount of DNA binding site. Prepare three control DNA samples at the same time. To two of these samples, add binding buffer in place of protein. The third control sample is identical to the experimental sample.

**2** | Incubate the samples at a temperature and for a duration that allows binding of protein to DNA to reach equilibrium.

**3** | Bring the samples slowly to room temperature.

**4** | Set aside two of the control samples: the one that contained protein, and the one that did not. Continue the procedure at Step 7 for these two control samples.

**5** | To initiate the hydroxyl radical cleavage reaction for the experimental sample and the remaining control sample, carefully spot 1 μl each of the following three solutions as separate drops on the wall of the Eppendorf tube: 10 mM sodium ascorbate, 0.6% H<sub>2</sub>O<sub>2</sub>, and iron(II)EDTA (1 mM Fe(II)/2 mM EDTA).

**▲ CRITICAL STEP** Reagents used to carry out hydroxyl radical footprinting should not be premixed before being added to the protein-DNA sample. Premixing the reagents initiates the Fenton reaction prematurely, expending hydroxyl radicals before the target DNA is added.

**6** | Using a clean pipette tip, mix the three individual reagent drops together on the wall of the Eppendorf tube and then quickly add to the sample. Immediately begin timing the reaction. Let the reaction proceed for 4 min, and then quench by adding 1 μl of 100 mM thiourea. *Note:* The reaction time can be varied (1–5 min) to optimize the extent of DNA cleavage. Separate reactions should be carried out in parallel to determine the reaction time that gives optimum cleavage.

**▲ CRITICAL STEP** The Fenton reaction is initiated by mixing the hydroxyl radical reagents, which generates the radical species necessary for cleavage of the DNA backbone. It is important to stop the cleavage reaction by adding the thiourea stop solution, which quenches the reaction by reacting with any remaining hydroxyl radical.

### DNA sample cleanup ● TIMING day 1, 6–8 h

**7** | Extract the reaction mixture (and each control sample) with 200 μl of a 1:1 phenol:chloroform mixture, to remove the DNA-binding protein. Retain the aqueous layer, which contains the radiolabeled DNA cleavage products. Follow standard protocols for phenol:chloroform extraction<sup>57</sup>.

**8** | Precipitate the DNA by adding 3 M sodium acetate (cold) to the aqueous layer from the phenol:chloroform extraction (see Step 7) so that the final concentration of sodium acetate is 0.3 M. Add chilled 100% ethanol to a final concentration of 70%. Place the sample in a dry ice/acetone bath for 2 h.

## PROTOCOL

- 9| Spin the precipitated DNA sample at 13,000*g* for 30 min at 4 °C in an Eppendorf microcentrifuge (6 cm rotor radius).
- 10| Discard the supernatant carefully without disturbing the pellet, which contains the DNA. This is best done by gentle vacuum aspiration of the supernatant using a syringe needle attached to a water aspirator. Check the tube using a Geiger counter to ensure that a substantial fraction (> 50%) of the radioactivity is retained in the pellet after discarding the supernatant.
- 11| Add 100 µl of chilled 70% vol/vol ethanol to the pellet and place the sample in a dry ice/acetone bath for 1 h. Spin the sample at 13,000*g* for 30 min at 4 °C in an Eppendorf microcentrifuge. Remove the supernatant as outlined in Step 10.
- 12| Wash the pellet with 400 µl of chilled 70% ethanol and spin at 13,000*g* for 15 min at 4 °C in an Eppendorf microcentrifuge. Remove the supernatant gently and dry the sample thoroughly in a SpeedVac concentrator for 30 min.
- 13| Resuspend the dried DNA in 10 µl of 2× gel loading dye.  
■ **PAUSE POINT** Store the resuspended DNA sample at −20 °C. Gel electrophoresis may be carried out the next day. Samples should not be stored for > 3 d at −20 °C following the hydroxyl radical footprinting reaction.

### Denaturing gel electrophoresis ● TIMING day 2, 5 h

- 14| Siliconize the larger of the two glass plates using Sigmacote as outlined in EQUIPMENT SETUP.
- 15| Assemble the gel plate sandwich with spacers and comb (0.33-mm thickness).
- 16| Initiate gel polymerization by adding TEMED and 10% wt/vol APS to the polyacrylamide gel mixture. See **Table 2** for the amount of TEMED and APS that is to be added for a given amount of gel mixture, and **Table 1** for the percent acrylamide to use for optimum separation. Pour the gel. Allow to polymerize for ~ 1 h.

### ? TROUBLESHOOTING

- 17| Mount the gel in the gel electrophoresis apparatus (see EQUIPMENT SETUP). Prerun the gel at constant power (65 W) for 30 min.  
▲ **CRITICAL STEP** The power at which the gel runs should be adjusted so that the surface temperature of the gel plates does not exceed 55 °C. Monitor the gel temperature by placing an adhesive temperature strip on one of the gel plates.
- 18| Heat the DNA samples (from Step 13 above) at 95 °C for 3 min to denature the DNA strands. Load 3 µl of each sample into a lane of the prerun gel.
- 19| Electrophorese at constant power (65 W) for sufficient time to resolve the region of interest. The bromphenol blue and xylene cyanol dyes that are included in the gel loading dye provide convenient visible markers for estimating the optimum time for electrophoresis.

### Drying the gel ● TIMING day 2, 2 h

- 20| After electrophoresis, allow the gel to cool to room temperature and remove from the apparatus.
- 21| Place the gel sandwich on a lab bench and carefully remove one glass plate (the plate that was siliconized) from the gel assembly. Without delay, place a large piece of Whatman chromatography paper on the exposed gel.
- 22| Press the chromatography paper to the gel evenly throughout to ensure that the gel sticks to the paper.
- 23| Invert the assembly. Pull the chromatography paper away from the glass plate slowly and carefully, peeling the gel along with the paper.
- 24| Cover the exposed side of the gel with Saran wrap. Take care to avoid wrinkles between the gel and the Saran wrap. The presence of wrinkles can lead to inefficient gel imaging during exposure to the phosphor screen.
- 25| Dry the gel using the gel dryer for ~ 2 h at 80 °C. The gel drying conditions should be optimized for the acrylamide percentage of the gel and the gel dryer that is used. The drying time may be varied by changing the temperature and the degree of vacuum that is applied to the gel.

### Gel exposure, scanning and image processing ● TIMING day 2, 2–8 h

- 26| Place the dried gel in an exposure cassette at room temperature and expose to a phosphor screen. The exposure time can vary from 1 to 6 h depending upon the radioactivity of the DNA sample.
- 27| Scan the phosphor screen using a phosphorimager (Storm 860 or equivalent) to produce a scan file (gel extension).
- 28| Process the gel file by importing it into SAFA (a computer program for semiautomated footprint analysis)<sup>56</sup>. SAFA is used for lane and band assignment and for quantitation of gel bands.

**29** Nucleotides that constitute the footprint are identified by comparing the missing bands (caused by protein binding to DNA) in the otherwise-uniform cleavage pattern to the bands resulting from control reactions (the Maxam–Gilbert guanine-specific sequencing reaction and the hydroxyl radical cleavage reaction of free DNA) (see **Figs. 1** and **2**).

### ? TROUBLESHOOTING

#### ● TIMING

Steps 1–6, hydroxyl radical footprinting reaction (day 1): 1–2 h

Steps 7–13, DNA sample cleanup (day 1): 6–8 h

Steps 14–19, denaturing gel electrophoresis (day 2): 5 h

Steps 20–25, drying the gel (day 2): 2 h

Steps 26–29, gel exposure, scanning and image processing (day 2): 2–8 h

### ? TROUBLESHOOTING

#### Step 16: air bubbles and pockets form upon pouring the sequencing gel

Bubbles form when gel plates have not been adequately cleaned. Gel plates should be soaked in 1 M NaOH overnight, washed with a mild soap solution, thoroughly rinsed with dH<sub>2</sub>O, rinsed with 0.1 M HCl and rinsed again with dH<sub>2</sub>O. Dry the plates completely using Kimwipes. Spray plates with 100% ethanol and wipe dry using Kimwipes. Repeat the ethanol spray/wipe step three times. Apply 1 ml of Sigmacote to the larger plate immediately before pouring the gel. Acrylamide solutions must be filtered and degassed before polymerization.

#### Step 29: DNA strand cleavage is observed within the expected footprint region

Failure to observe a clear footprint can sometimes occur because the mode of protein binding to DNA does not interfere with hydroxyl radical attack. For example, the bZIP protein C/EBP binds to DNA by inserting extended  $\alpha$  helices deep into the major groove of DNA, resulting in little protection of the sugar–phosphate backbone from hydroxyl radical cleavage, and so only a very weak footprint is observed<sup>58</sup>.

More typically, the lack of a footprint can be the consequence of one or more of the hydroxyl radical reagents interfering with binding of the protein to DNA. If this is the case, a DNA–protein complex that is subjected to the hydroxyl radical footprinting reaction will show cleavage bands in a region where a footprint is expected, because the protein is not bound to the DNA molecule during the experiment. For example, we found that 0.03% H<sub>2</sub>O<sub>2</sub> inhibits the binding of the zinc finger protein TFIIIA<sup>41</sup> and the ‘copper-fist’ protein CUP2 (ref. 53), probably by interfering with metal binding to the protein. To test for this possibility, carry out a set of DNase I footprinting experiments to optimize the concentration of each of the hydroxyl radical cleavage reagents<sup>53</sup> (see **Box 2**).

#### Step 29: low, or no, DNA cleavage is observed

The most common cause of low cleavage is inactive (oxidized) sodium ascorbate. Ascorbate is used to reduce the oxidized iron(III) product of the cleavage reaction (see Eq. 1) to permit additional rounds of hydroxyl radical production. If the ascorbate is oxidized and thus incapable of reducing iron(III), the cleavage reaction will be sluggish. Glycerol or other hydroxyl radical scavengers can also inhibit cleavage if they are present in the footprinting reaction mixture. To increase cleavage, try the following remedies:

- Prepare fresh reagents (sodium ascorbate, iron(II)EDTA and H<sub>2</sub>O<sub>2</sub>) (see REAGENT SETUP).
- Protect reagents from light by covering reagent stock tubes in aluminum foil.
- Completely thaw and ensure that reagent solutions are at room temperature before adding them to the DNA–protein sample.
- Sodium ascorbate, iron(II)EDTA, and H<sub>2</sub>O<sub>2</sub> are introduced as individual drops on the wall of the reaction tube. After the drops are mixed together on the side of the sample tube, the reagent mixture must be added rapidly to the DNA–protein sample.
- Protein samples often are stored in glycerol, which can inhibit the hydroxyl radical cleavage reaction. If possible, dialyze the protein into a buffer containing a low concentration of (ideally, no) glycerol.
- Tris and HEPES buffer at high concentration can inhibit the hydroxyl radical cleavage reaction. The concentrations of these buffers should be < 10 mM.
- The concentration of sodium ascorbate should be several times that of iron(II)EDTA (10× is recommended) to maintain a catalytic cycle.

#### Step 29: the hydroxyl radical cleavage pattern is distorted or smeared

- The presence of excess salt in the DNA sample (see Step 12), due to inadequate removal of sodium acetate or other salts following ethanol precipitation, can lead to distortion of DNA migration during electrophoresis, manifested as lane narrowing near the bottom of the gel. The resulting distorted bands are difficult to visualize and quantify. To solve this problem, repeatedly rinse the DNA sample with chilled 70% ethanol (Step 12) to remove excess salt.
- Excessive gel heating can cause band smearing. Monitor gel temperature by placing thermometer strips on the surface of the glass plate. Do not exceed a surface temperature of 55 °C.

## BOX 2 | PROCEDURE TO OPTIMIZE THE HYDROXYL RADICAL FOOTPRINTING REACTION WHEN CLEAVAGE REAGENT(S) INTERFERE WITH PROTEIN BINDING TO DNA

1. Incubate radiolabeled DNA containing the binding site with the DNA-binding protein for 15 min, in four separate sample tubes.
2. To three of the samples, add one each of the hydroxyl radical reagents [iron(II)EDTA, sodium ascorbate or hydrogen peroxide] and incubate for 5 min. Also incubate a fourth, mock-treated, sample.
3. Perform DNase I digestion on the four samples, electrophorese the products on a denaturing gel, and image the gel, according to the standard procedure (see PROCEDURE).
4. If a DNase footprint is not apparent in a sample that was treated using one of the hydroxyl radical reagents, reduce the concentration of the interfering reagent(s) and repeat the assay.
5. When reagent concentrations have been found that do not interfere with the DNase footprint, repeat the hydroxyl radical footprinting experiment using these new (lower) reagent concentrations. The concentrations of the noninterfering reagents can be increased, if need be, to compensate for the lower concentration of the interfering reagent, so that adequate DNA cleavage is achieved in the hydroxyl radical footprinting reaction<sup>53</sup>.

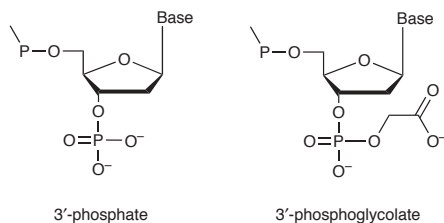
### ANTICIPATED RESULTS

The nucleotides that the protein covers when it is bound to DNA are apparent in the gel image as blanks (missing bands). These missing bands are called the footprint. **Figure 1** depicts in schematic form the gel image that results from hydroxyl radical cleavage of DNA with and without bound protein. **Figure 2** shows an autoradiograph of an actual denaturing gel<sup>59</sup> on which was separated the products of a hydroxyl radical footprinting experiment. Owing to the single-nucleotide resolution afforded by hydroxyl radical footprinting, the precise size of the protein binding site as well as its sequence is revealed by comparing footprinted regions to control samples and sequencing ladders.

One subtlety of footprint analysis lies in assigning the nucleotide sequence of the footprint. Hydroxyl radical cleavage of DNA produces strand breaks that are the result of abstraction of a hydrogen atom from a deoxyribose in the DNA backbone (**Fig. 3**). The deoxyribose residue that was the site of hydroxyl radical attack is destroyed by the reaction, and so the product DNA fragment is actually 1 nt shorter than if it were terminated by the residue that was attacked by the radical. It is fortunate that exactly the same thing happens with the Maxam–Gilbert guanine-specific sequencing reaction that is the recommended source of markers for assigning the footprint. Maxam–Gilbert chemistry also produces a DNA product fragment that is 1 nt shorter than the base with which a particular gel band is identified<sup>55</sup>. For example, a band in a Maxam–Gilbert sequencing lane that is assigned to a guanine that is 50 nt from the 5' end of a DNA molecule actually is produced by a DNA fragment 49-nt long. In addition, both Maxam–Gilbert chemistry and hydroxyl radical cleavage produce DNA strands terminated by a phosphate group (**Fig. 3**), so the DNA fragments produced using both methods have identical electrophoretic mobilities. The fortunate correspondence between the Maxam–Gilbert sequencing pattern and the hydroxyl radical cleavage pattern makes it a simple matter to assign the sequence of a hydroxyl radical footprint—there is a one-to-one correspondence between a band in the Maxam–Gilbert lane and a band in the hydroxyl radical footprint lane. (This discussion should make it evident that if other kinds of DNA fragments are used as electrophoretic markers to assign a footprint, it is incumbent on the experimenter to understand how the marker fragments were produced.)

In many cases, small patches of protection from hydroxyl radical cleavage are seen within a larger binding site<sup>10</sup> (e.g., as determined by DNase footprinting). This pattern of protection shows directly that the protein is bound to only one face of the DNA molecule, thus providing detailed structural information on the protein–DNA complex that is not available from other footprinting methods<sup>4</sup>.

Multiple bands at one or more nucleotide positions on the gel sometimes are observed because the highly reactive hydroxyl radical is capable of abstracting more than one of the deoxyribose hydrogen atoms<sup>60</sup>. For example, abstraction of a C4' hydrogen atom from the deoxyribose produces two distinct strand termini at the 3' end (**Fig. 4**): a 3'-phosphate terminus and a 3'-phosphoglycolate terminus<sup>12</sup>.



**Figure 4** | Products of hydroxyl radical cleavage that result from the abstraction of a 4'-hydrogen atom from the deoxyribose sugar. At the 3' end of the strand break, two termini are produced: a 3'-phosphate and a 3'-phosphoglycolate.

Observation of multiple cleavage bands at a particular position within the DNA sequence can pose a challenge to quantification. In practice, this is only a problem when very high-resolution electrophoresis is used to separate cleavage products<sup>60</sup> (e.g., a  $\geq 20\%$  polyacrylamide denaturing gel with a short DNA molecule). More typically, longer DNA molecules are used in the experiment. The lower-percentage gel that is employed for separation of the cleavage products (see **Table 1**) does not resolve the different strand termini. If necessary, accurate quantitation of closely spaced, overlapping, and imperfectly shaped bands can be carried out by band deconvolution, which has been incorporated into some software packages for gel analysis<sup>56,61</sup>.

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